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[SEAL- MINISTRY OF INDUSTRY
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I hereby certify that the attached documents are an exact copy of the PATENT of INVENTION application number 200300708, having a filing date in this Institution of 26 March, 2002.

Madrid, 7 May, 2004

Patent and Technological Information
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IN VITRO METHOD FOR DETECTING CARCINOMAS

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(16) NOTIFICATION OF GRANT FEE PAYMENT

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COMPLEMENTARY INFORMATION SHEET

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ABSTRACT AND GRAPHIC

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ABSTRACT (150-word maximum)

"IN VITRO METHOD FOR DETECTING CARCINOMAS"

The present invention relates to an *in vitro* method for detecting the presence of a bladder transitional cell carcinoma in an individual, for determining the stage or the severity of said carcinoma in the individual or for monitoring the effect of the therapy administered to an individual with said carcinoma, which comprises a) the detection and/or quantification of the FGFR3 protein, of the mRNA of the FGFR3 gene or the corresponding cDNA in a sample of said individual, and b) the comparison of the amount of FGFR3 protein, of the amount of mRNA of the FGFR3 gene or of the amount of the corresponding cDNA detected in a sample of an individual, with the amount of FGFR3 protein, with the amount of the mRNA of the FGFR3 gene or with the amount of the corresponding cDNA detected in the samples of control individuals or in previous samples of the same individual or with the normal reference values; as well as to the screening, identification, development and evaluation of the efficacy of compounds for the therapy of said bladder transitional cell carcinoma for the purpose of developing new medicinal products, as well as agents inhibiting the expression and/or activity of the FGFR3 protein.

GRAPHIC

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54. TITLE OF THE INVENTION

"IN VITRO METHOD FOR DETECTING CARCINOMAS"

57. ABSTRACT

"IN VITRO METHOD FOR DETECTING CARCINOMAS"

The present invention relates to an *in vitro* method for detecting the presence of a bladder transitional cell carcinoma in an individual, for determining the stage or the severity of said carcinoma in the individual or for monitoring the effect of the therapy administered to an individual with said carcinoma, which comprises a) the detection and/or quantification of the FGFR3 protein, of the mRNA of the FGFR3 gene or the corresponding cDNA in a sample of said individual, and b) the comparison of the amount of FGFR3 protein, of the amount of mRNA of the FGFR3 gene or of the amount of the corresponding cDNA detected in a sample of an individual, with the amount of FGFR3 protein, with the amount of the mRNA of the FGFR3 gene or with the amount of the corresponding cDNA detected in the samples of control individuals or in previous samples of the same individual or with the normal reference values; as well as to the screening, identification, development and evaluation of the efficacy of compounds for the therapy of said bladder transitional cell carcinoma for the purpose of developing new medicinal products, as well as agents inhibiting the expression and/or activity of the FGFR3 protein.

IN VITRO METHOD FOR DETECTING CARCINOMAS

Field of the Invention

The present invention relates to an *in vitro* method for detecting the presence of a bladder transitional cell carcinoma in an individual, for determining the stage or the severity of said carcinoma in the individual, or for monitoring the effect of the therapy administered to an individual with said carcinoma; to the screening, identification, development and evaluation of the efficacy of compounds for the therapy of said bladder transitional cell carcinoma for the purpose of developing new medicinal products; as well as to agents that inhibit the expression and/or activity of the FGFR3 protein.

Background of the Invention

Despite all the advances that have been achieved during the last 20 years, cancer is still one of the leading causes of mortality worldwide. Bladder transitional cell carcinoma is the most common cancer of the urinary tract; it is also the fourth most common cancer in men and the eighth most common in women. Based on data from the International Agency for Research on Cancer, GLOBOCAN, of the year 2000, every year more than 136,000 new cases are diagnosed in Europe, 13,000 in Japan and 56,000 in North America. More than 3-4 times this number of patients are treated and monitored at hospitals every year; and more than 49,000, 4,500 and 12,000 deaths are due to bladder cancer every year in Europe, Japan and North America, respectively.

Transitional cell carcinoma of the bladder is the most common type of bladder cancer; it comprises more than 90% of all cases. The remaining cases are squamous cell carcinomas (7%), adenocarcinomas (2%), and undifferentiated carcinomas (1%).

Tumour grade and clinicopathological stage are the best prognostic indicators of bladder transitional cell carcinoma.

Bladder tumours are graded cytomorphologically from G1 to G3 in decreasing state of differentiation and increasing aggressiveness of the disease according to the World Health Organization (WHO). With respect to stage or invasivity, TCCs of the bladder are classified as superficial with and without involvement of the lamina propria (Ta and T1), deep layer infiltrating (T2 to T4), or the uncommon carcinoma *in situ* or tumour *in situ* (TIS). Low-grade (G1) tumours are usually confined to the mucosa or infiltrate superficial layers (stage Ta and T1). Most high-grade tumours are detected at least at T1 stage (invading the lamina propria). Approximately 75% of the diagnosed cases of bladder transitional cell carcinoma are superficial. The remaining 25% are muscle invasive at the time of diagnosis. The importance of clinically separating superficial and infiltrating tumours stems from the need to perform radical cystectomy, with lymphadenectomy and bladder reconstruction in cases showing infiltration beyond the muscle layer. Tumours diagnosed in stages Ta and T1 allow the organ to be preserved and can be treated by means of transurethral resection and in some cases chemotherapy or intravesicular immunotherapy.

Patients with superficial bladder transitional cell carcinoma have a good prognosis but have a 70% risk of locoregional recurrence; these patients have to be monitored for recurrence after treatment, following different protocols depending on the hospital, although the most frequent method is evaluation by the urologist every 3 months for the first 2 years, every 6 months for the following 2 years and every year thereafter. In spite of the high risk of recurrence, Ta tumours tend to be low grade and only 10-15% will progress to muscle invasive after 2 years; the percentage of T1 tumours that progresses to stage T2 is higher (30-50%). Patients with invasive bladder transitional cell carcinoma have a poor prognosis; 50% of these patients at stage T2 or higher develop distant metastases during the two years after diagnosis, and

69% of them die within 5 years. New early diagnosis systems are needed given that 80-90% of patients in stage T2 or higher are diagnosed *de novo* at this highly aggressive stage and not in previous stages (de Vere White, R.W. and Stapp, E., Oncology, 1998, 12:1717-1723).

Currently, the best diagnosis system for bladder transitional cell carcinoma in patients presenting symptoms such as haematuria or dysuria, in the absence of infection, is cytoscopy. Based on statistical data of incidence and recurrence, it has been estimated that more than 500,000 cystoscopies are performed annually in the USA (van Rhijn, B.W.G., et al., Cancer Res., 2001, 61:1265-1268). Flexible cytoscopes are used to make the technique less aggressive, but it remains invasive and requires some form of anaesthesia. The non-invasive technique of choice for the diagnosis of transitional cell cancer of the bladder consists of identifying neoplastic cells through the morphological examination of the cells in urine samples (Loh, C.S., et al., Br. J. Urol., 1996, 77:655-658). Cytology is currently used to follow up patients diagnosed with and treated for bladder carcinomas. On the other hand, urine cytology can detect neoplasias *in situ* without macroscopic manifestation and carcinomas of the upper urinary tract, i.e. ureter, pelvis and renal, that are not accessible for any endoscopic process (Lotan, Y. and Roehrborn, J. Urol., 2002, 167:75-79). Nevertheless several studies have shown that cytology has a very low sensitivity and does not identify 50% of the tumours (Boman, H., et al., J. Urol., 2002, 167:80-83); currently, there is no non-invasive method available for detecting bladder transitional cell carcinoma with high sensitivity and specificity (Boman, H., et al., J. Urol., 2002, 167:80-83). These non-invasive methods would allow routine screening procedures such as the early detection of any type of transitional carcinoma, including of the upper urinary tract, both *de novo* or in evaluating recurrences after treatment, and

could even identify incipient infiltrating tumours or those at a higher risk of developing aggressive disease.

Alteration of gene expression levels is closely associated with uncontrolled cell growth and dedifferentiation, common features of all types of cancers. The expression levels of the so-called "tumour suppressor genes", which act by preventing malignant cell growth, are repressed in tumour cells; and the expression levels of the so-called "oncogenes", which act by inducig malignant growth, are elevated in tumour cells. Many of these genes have been associated with development of bladder transitional cell carcinoma, including Rb, p53, p16, p14ARF, cyclin D1 (Fujimoto, K., et al., Cancer Res., 1998, 52:1393-1398; Grossman, B.H., et al., Clin. Cancer Res., 1998, 8:829-834; Balazs, M., et al., Genes Chromosomes Cancer, 1997, 19:84-89). The alteration in the expression of said genes could be used as a diagnostic marker of bladder transitional cell carcinoma; among these potential markers, nuclear matrix protein NMP22 (Soloway, M.S., et al., J. Urol., 1996, 156:363-367; Casella, R., et al., J. Urol, 2000, 164:1926-1928), Hyaluronic Acid and Hyaluronidase (Pham, H.T., et al., Cancer Res., 1997, 57:778-783; Hautmann, S.H., et al., J. Urol., 2001, 165:2068-2074), Basement Membrane Complexes (BTA) (Pode, D., et al., J. Urol., 1999, 161:443-446; Thomas, L., et al., Clin. Chem, 1999, 45:472-477), Carcinoembryonic antigen (CEA) (Halim, A.B., et al., Int. J. Biol. Markers, 1992; 7:234-239), Uroplakin II (Wu, X.R., et al., Cancer Res., 1998; 58:1291-1297), Scatter Factor/Hepatocyte Growth Factor (SF/HGF) (Gohji, K., et al., J. Clin. Oncol., 2000; 18:2963-2971), Mammary tumour 8-Ka Protein (MAT-8) (Morrison, B.W., et al., J. Biol. Chem., 1995, 270:2176-2182), Telomerase (Neves, M., et al., J. Urol., 2002, 167:1276-1281), proteins of the keratin/cytokeratin family such as cytokeratin 20 (Buchumensky, V., et al., J. Urol., 1998, 160:1971-1974), and cytokeratin 18 (Sánchez-Carbayo, M., et al., Clin. Cancer Res., 2000, 6:3585-3594), have been

proposed. However, there is no marker for the early diagnosis of bladder transitional cell carcinoma which has been proven useful in clinical trials (Miyake, H., et al., J. Urol., 2002; 167:1282-1287). Many of the genes involved in the initiation and progression of bladder transitional cell carcinoma are still unknown; the identification of differentially expressed genes in bladder transitional cell carcinoma could lead to the identification of biological markers, which could be of significant value for the diagnosis, prognosis and treatment of this disease.

Once bladder transitional cell carcinoma has been diagnosed, transurethral resection is carried out to treat superficial papillary tumours; TIS and T1 are treated, in addition to applying resection with Bacillus-Calmette Guerin (BCG) in the form of intravesical instillations. If the cancer is muscle invasive, the patient is treated by radical cystectomy; if the patient does not tolerate this surgery, radiation therapy or chemotherapy is used.

69% of the patients with muscle invasive bladder transitional cell carcinoma die within five years after diagnosis, even after receiving treatment. Alternative therapeutic approaches are necessary to treat muscle invasive bladder transitional cell carcinoma with a higher efficiency; also needed are alternative therapeutic approaches to treat low-grade tumours more efficiently than through surgery, or to complement surgery in order to avoid recurrence and progression of tumours to invasive stages.

Fibroblast growth factors (FGF) are a family of more than 90 proteins involved in the regulation of biological processes including cell proliferation, cell differentiation, cell growth, cell migration, morphogenesis, angiogenesis and tissue remodelling. FGFs bind with high affinity to cell surface receptors (FGF Receptors, or FGFRs) that have tyrosine kinase activity. kinases are a family of proteins which effect phosphorylate other proteins and play a key role in the

regulation of many cell processes (Hanks S.K., et al., Science 1988, 241, 42-52). When the FGF ligand binds to FGFR, the FGFR is converted into a dimeric active form that autophosphorylates in the kinase domain; then the activated FGFR binds and phosphorylates other effector proteins, thus starting a signal transduction cascade from the cell surface to the nucleus (Crews and Erikson. Cell. 1993. 74:215-217). The loss of regulation of the growth factor signalling cascade is a frequent occurrence in tumour processes.

Four FGFRs have been identified to date: FGFR1, also called Flg, fms-like gene, flt-2, bFGFR, N- bFGFR or Cek1; FGFR2, also called Bek, bacterial-expressed kinase, KGFR, Ksam, KsamI and Cek3; FGFR3, also called Cek2; and FGFR4. All mature FGFRs share a common structure consisting of an amino terminal signal peptide, three extracellular immunoglobulin-like domains (Ig I domain, Ig II domain, Ig III domain), with an acidic region between the Ig I and II domains (the acidic box domain), a transmembrane domain, and intracellular kinase domains (Ullrich, A., and Schlessinger, J.,, Cell 61:203, 1990; Jonson, D.E., and Williams L.T., Adv. Cancer Res, 1992, 60:1-41). The distinct FGFR isoforms have different affinities for the different ligands; thus FGF8 and FGF9 have the highest affinity for FGFR3 (Chellaiah, A., et al. J Biol. Chem., 1999, 274:34785-34794).

Specific point mutations that lead to the activation of its tyrosine kinase activity and to different syndromes related to bone development have been associated in *FGFR3* (Chen, H., et al. J. Clin. Invest., 1999, 104(11):1517-1525).. Mutations in FGFR3 have also been detected in multiple myelomas (10-25% of tumours. Plowright, E.E., et al. Blood, 2000, 95:992-998; Chesi, M., et al. Blood, 2001; 97:729-736; Soverini, S., et al. Haematologica, 2002, 87:1036-1040; Pollett, J.B., et al. Blood, 2002, 100:3819-3821), in cervical carcinomas (3.5-25% of tumours. Sibley, K., et al. Oncogene, 2001, 20:4416-4418; Dai, H., et al. Anal Cell Pathol., 2001,

23:45-49) and in transitional cell carcinoma of the bladder (Cappellen, D. et al. Nat .Genet. 1999, 23:18-20; Sibley, K., et al., Oncogene, 2001, 20:686-691; Sibley, K., et al., Oncogene, 2001, 20:4416-4418; Billerey, C., et al., Am. J. Pathol., 2001, 158:1955-1959) Activating mutations of the function of *FGFR3* were detected in 40-50% of transitional carcinomas of the bladder; the incidence was significantly higher, 80%, in superficial tumours than in invasive tumours; and the recurrence percentages were significantly lower in tumours with a mutation in FGFR3 (Kimura, T., et al., Cancer, 2001, 92:2555-2561; van Rhijn, B.W.G., et al., Cancer Res., 2001, 61:1265-1268).

The authors of the present invention have discovered, after thorough research, that the expression of the *FGFR3* gene and concentration of the FGFR3 protein are elevated in biopsies of transitional cell carcinomas of the bladder; and, furthermore, that the treatment of bladder tumour cells expressing high levels of FGFR3, with an antibody specific against the FGFR3 protein, causes the inhibition of cell proliferation of bladder tumour cell lines.

The present invention, therefore, provides a highly sensitive *in vitro* method for detecting the presence of a bladder transitional cell carcinoma in an individual, for determining the stage or the severity of said carcinoma in the individual or for monitoring the effect of the therapy administered to an individual with said carcinoma. Likewise, the present invention provides targets or tools for the screening, identification, development and evaluation of the efficacy of compounds for the therapy of bladder transitional cell carcinoma, particularly for the treatment of tumours, as neoadjuvant before resection or as adjuvant after resection, for the purpose of reducing the possibilities of recurrence and progression. Finally, the invention provides agents characterised in that they inhibit the expression and/or the activity of the FGFR3 protein for the treatment of bladder

transitional cell carcinoma.

Object of the Invention

The main object of the present invention is the development of an *in vitro* method for detecting the presence of a bladder transitional cell carcinoma in an individual, for determining the stage or the severity of said carcinoma in the individual or for monitoring the effect of the therapy administered to an individual with said carcinoma.

Another object of the present invention is an *in vitro* method for screening for, identifying, developing and evaluating the efficacy of compounds for the therapy of bladder transitional cell carcinoma.

An additional object of the invention lies in the use of sequences derived from the *FGFR3* gene for the diagnosis and prognosis *in vitro* of bladder transitional cell carcinoma, as well as to screen for, identify, develop and evaluate the efficacy of compounds for the therapy of said carcinoma.

Another object of the present invention consists of providing agents characterized in that they inhibit the expression and/or the activity of the FGFR3 protein, for the treatment of bladder transitional cell carcinoma.

Finally, another object of the invention is a pharmaceutical composition comprising one or several therapeutic agents together with a pharmaceutically acceptable excipient for the treatment of bladder transitional cell carcinoma.

Description of the Drawings

Figure 1 shows the results of the Western Blot analysis of the expression of the FGFR3 protein in samples of human bladder. The following was analysed: three samples of normal bladder (sample numbers 46, 55 and 63), six samples of low-grade superficial (G1, Ta) transitional cell carcinoma of the bladder (sample numbers 48, 49, 50, 53, 56 and 59), three samples of high-grade lamina propria invasive (G3, T1) transitional cell carcinoma (sample numbers 57, 61 and 67),

four samples of high-grade detrusor muscle invasive (G3, T2) carcinomas (sample numbers 47, 51, 58 and 60) and two samples of unknown grade (sample numbers 54 and 62). The amount of total extract of protein was 20 µg in all cases. The membranes were developed with anti-FGFR3 antibody (A) or with anti-actin as loading control (B). The receptor appeared in the form f several immunoreactive bands of different molecular weight: 135 kDa, corresponding to the fully glycosylated form; 85 kDa, corresponding to the intracellular non-glycosylated form, and bands of intermediate molecular mass (110-110 kDa), which correspond to different degrees of glycosylation of FGFR3. Immunoreactive bands of lower molecular weight (50 kDa) were also detected that may have resulted from the proteolytic degradation thereof.

Figure 2 shows the results of a western blot analysis of the expression of the FGFR3 protein in the cell line of human bladder transitional cell carcinoma, RT-112. A sample of normal human bladder (sample number 46) was added as negative control, and a sample of bladder tumour (sample number 53) was added as positive control. The amount of protein loaded was 20 µg in all cases.

Figure 3 shows the effects of the anti-FGFR3 (blue bars) and anti-β2 microglobulin (red bars) antibodies on the growth of RT-112 bladder transitional carcinoma cells in serum-free medium. The cells were seeded in 96-well plates and were treated with the antibodies for 24 or 48 h. The growth is expressed in relation to the controls (without antibody). The points are a mean of 6 replicas, the vertical lines represent the standard deviation.

Detailed Description of the Invention

To facilitate the understanding of the present patent application, the meaning of some terms and expressions in the context of the invention are set forth below:

The terms "subject" or "individual" refer to members of

species of mammalian animals and includes, but is not limited to, domestic animals, primates and humans; the subject is preferably a male or female human of any age or race

The term "cancer" refers to the disease that is characterised by an uncontrolled proliferation of abnormal cells capable of invading adjacent tissues and spreading to distant organs.

The term "bladder transitional cell carcinoma" refers to any malignant proliferative disorder of bladder transitional epithelial cells.

The term "tumour" refers to any abnormal mass of tissue generated by a benign (non cancerous) or malignant (cancerous) neoplastic process.

The term "gene" refers to a molecular chain of deoxyribonucleotides which encodes a protein.

The term "DNA" refers to deoxyribonucleic acid. A DNA sequence is a sequence of deoxyribonucleotides.

The term "cDNA" refers to a nucleotide sequence complementary to an mRNA sequence.

The term "RNA" refers to ribonucleic acid. An RNA sequence is a sequence of ribonucleotides.

The term "mRNA" refers to messenger ribonucleic acid, which is the fraction of total RNA which is translated into proteins.

The term "mRNA transcript of" refers to the transcription of the gene (DNA) into mRNA, as a first step for the gene to be expressed and translated into protein.

The term "sequence of nucleotides" or "nucleotide sequence" refers either to a sequence of ribonucleotides (RNA) or a sequence of deoxyribonucleotides (DNA).

The term "protein" refers to a molecular chain of amino acids, with a biological activity.

The terms "peptide" and "polypeptide" refer a protein fragment. The terms "protein" and "peptide" are used indistinguishably.

The phrase "high levels" means that the levels measured in patients with bladder transitional cell carcinoma are higher than the levels measured in a control population of individuals with no history of bladder transitional cell carcinoma.

The term "sensitivity" refers to the detection of false negatives (negative diagnosis of bladder transitional cell carcinoma when the patient does have bladder transitional cell carcinoma); a sensitivity of 100% means there are no false negatives.

The term "specificity" refers to the detection of false positives (positive diagnosis of bladder transitional cell carcinoma when the patient does not have bladder transitional cell carcinoma); a specificity of 100% means there are no false positives.

The term "antibody" refers to a glycoprotein that exhibits a specific binding activity for a particular protein, referred to as "antigen". The term "antibody" comprises monoclonal antibodies, or polyclonal antibodies, either intact or fragments thereof; and includes human antibodies, humanised antibodies and antibodies of non-human origin. "Monoclonal antibodies" are homogeneous populations of highly specific antibodies, that are directed against a single antigenic site or "determinant". "Polyclonal antibodies" include heterogeneous populations of antibodies that are directed against different antigenic determinants.

The term "epitope", as it is used in the present invention, refers to an antigenic determinant of a protein, which is the sequence of amino acids of the protein that a specific antibody recognises.

The term "solid phase", as it is used in the present invention, refers to a non-aqueous matrix to which the antibody can bind. Examples of materials for the solid phase include glass, polysaccharides, for example agarose, polyacrylamide, polystyrene, polyvinyl alcohol and silicones.

Examples of solid phase forms are the well of an assay plate or a purification column.

The term "oligonucleotide primer", as it is used in the present invention, refers to a nucleotide sequence that is complementary to a nucleotide sequence of the FGFR3 gene. Each primer hybridises with its target nucleotide sequence and acts as an initiation site for DNA polymerisation.

The term "probe", as it is used in the present invention, refers to a nucleotide sequence complementary to a nucleotide sequence derived from the FGFR3 gene that can be used to detect the nucleotide sequence derived from the FGFR3 gene.

The term "therapeutic target" refers to nucleotide or peptide sequences against which a drug or therapeutic compound can be designed and clinically applied.

The term "agonist" refers to any molecule that mimics the biological activity of the angonised molecule. Examples of agonist molecules include, among others, proteins, peptides, sequence variations of natural peptides and small organic molecules (of a molecular weight less than 500 Daltons).

The present invention is based on the discovery that both the gene expression of FGFR3 and the concentration of the FGFR3 protein are increased in bladder transitional cell carcinoma, and on the fact that the proliferation of bladder tumour cell lines is inhibited when they are treated with an antibody specific against the FGFR3 protein.

In this sense, the present invention first of all provides an *in vitro* method for detecting the presence of a bladder transitional cell carcinoma in an individual, for determining the stage or the severity of said carcinoma in the individual, or for monitoring the effect of the therapy administered to an individual with said carcinoma, which comprises:

a) the detection and/or quantification of the FGFR3 protein, of the mRNA of the *FGFR3* gene or the corresponding

cDNA in a sample of said individual, and

b) the comparison of the amount of FGFR3 protein, of the amount of mRNA of the *FGFR3* gene or of the amount of the corresponding cDNA detected in a sample of an individual, with the amount of FGFR3 protein, with the amount of the mRNA of the *FGFR3* gene or with the amount of the corresponding cDNA detected in the samples of control individuals or in previous samples of the same individual or with the normal reference values.

The method provided by the present invention is highly sensitive and specific and is based on the fact that subjects or individuals diagnosed with bladder transitional cell carcinoma present high levels of mRNA transcribed from the *FGFR3* gene (high expression levels of the *FGFR3* gene), or high concentrations of the protein encoded by the *FGFR3* gene (FGFR3 protein), in comparison with the corresponding levels in samples from subjects without a clinical history of bladder transitional cell carcinoma.

The present method comprises a step for obtaining the sample from the individual. Different fluid samples can be used such as, for example: urine, blood, plasma, serum, pleural fluid, ascitic fluid, synovial fluid, bile, semen or cerebrospinal fluid. The sample can also be bladder tissue that can be obtained by any conventional method, preferably by means of cystoscopy.

The samples can be obtained from subjects previously diagnosed or not diagnosed with bladder transitional cell carcinoma; or also from a subject receiving treatment, or who has previously been treated for bladder transitional cell carcinoma.

The present method furthermore comprises a step for extracting the sample, either to obtain the extract of proteins thereof or to obtain the extract of total RNA. One of these two extracts provides the working material for the next phase. The extraction protocols for total protein or total RNA

are well by the person skilled in the art (Chomczynski P. et al., Anal. Biochem., 1987, 162: 156; Chomczynski P., Biotechniques, 1993, 15: 532; Molina, M.A., et al., Cancer Res., 1999, 59: 4356-4362).

Any conventional assay can be used in the context of the invention to detect a bladder transitional cell carcinoma, provided that it measures *in vitro* the levels of mRNA transcribed from the *FGFR3* gene or its complementary cDNA, or the concentration of the FGFR3 protein, in samples collected from individuals to be analyzed and from control individuals.

Therefore, this invention provides a method for detecting the presence of a bladder transitional cell carcinoma in an individual, for determining the stage or the severity of said carcinoma in the individual, or for monitoring the effect of the therapy administered to an individual with said carcinoma, based either on measuring the concentration of the FGFR3 protein or on measuring the expression level of the *FGFR3* gene.

In the event that the aim is to detect the FGFR3 protein, the method of the invention comprises a first step for placing the extract of proteins of the sample in contact with a composition of one or more antibodies specific against one or more epitopes of the FGFR3 protein, and a second step for quantifying the complexes formed by antibodies and the FGFR3 protein.

There is a wide variety of immunological assays available to detect and quantify the formation of specific antigen-antibody complexes; numerous competitive and non-competitive protein binding assays have been described previously and a large number of these are commercially available.

Thus, the FGFR3 protein can be quantified with antibodies such as, for example: monoclonal antibodies, polyclonal antibodies, intact or recombinant fragments thereof, combibodies and Fab or scFv fragments of antibodies,

specific against the FGFR3 protein; these antibodies being human, humanised or of non-human origin. There are antibodies that bind specifically to the FGFR3 protein, which are commercially available. The antibodies that are used in these assays can be labelled or unlabelled; the unlabelled antibodies can be used in agglutination assays; the labelled antibodies can be used in a wide variety of assays. Marker molecules that can be used to label antibodies include radionuclides, enzymes, fluorophores, chemoluminescent reagents, enzymatic substrates or cofactors, enzymatic inhibitors, particles, colorants and derivatives.

There is a wide variety of well known assays that can be used in the present invention, which use unlabelled antibodies (primary antibody) and labelled antibodies (secondary antibodies); these techniques include but are not limited to the western blot, ELISA (Enzyme-Linked immunosorbent assay), RIA (Radioimmunoassay), Competitive EIA (Competitive enzyme immunoassay), DAS-ELISA (Double antibody sandwich-ELISA), immunocytochemical and immunohistochemical techniques, techniques based on the use of biochips or protein microarrays that include specific antibodies or assays based on colloidal precipitation in formats such as dipsticks. Other ways to detect and quantify the FGFR3 protein include affinity chromatography techniques, ligand binding assays or lectin binding assays.

The preferred immunoassays in the method of the invention is a double antibody sandwich ELISA assay. In this immunoassay any antibody, or combination of antibodies, specific against one or more epitopes of the FGFR3 protein, can be used. As an example of one of the many possible formats of this assay, a monoclonal or polyclonal antibody, or a fragment of this antibody, or a combination of antibodies, which coat a solid phase, are placed in contact with the sample to be analysed and incubated for a time and in conditions suitable for forming the antigen-antibody

complexes. After washing in suitable conditions to eliminate the non-specific complexes, an indicator reagent, comprising a monoclonal or polyclonal antibody, or a fragment of this antibody, or a combination of these antibodies, bound to a signal generating molecule, is incubated with the antigen-antibody complexes in suitable conditions and time. The presence of the FGFR3 protein in the sample to be analysed is detected and quantified, if present, by measuring the signal generated. The amount of FGFR3 protein present in the sample to be analysed is proportional to this signal.

In the event that the aim is to detect the mRNA or the cDNA corresponding to the *FGFR3* gene and not the protein, the method for evaluating *in vitro* the susceptibility of an individual to develop bladder transitional cell carcinoma of the invention has several different steps. Thus, after obtaining the sample and extracting the total RNA, the method of the invention for the detection of the mRNA or of the corresponding cDNA of the *FGFR3* gene, comprises a first step for amplifying the extract of total RNA or the corresponding cDNA synthesised by reverse transcription from the mRNA and a second step for quantifying the amplification product of the mRNA or of the cDNA of the *FGFR3* gene.

One example of amplification of mRNA consists of reverse transcription (RT) of the mRNA into cDNA, followed by Polymerase Chain Reaction (PCR), using oligonucleotide primers, the sequences of the primers used being 5'-GACGGTTTCC AGGGAGGGGC-3' and 5'-GTAACAGTACAGAA CGAACCAACTG-3'; PCR is a technique for the amplification of a certain (target) nucleotide sequence contained in a mixture of nucleotide sequences. In PCR, an excess of a pair of oligonucleotide primers is used that hybridise with complementary strands of the target nucleotide sequence. Then, an enzyme with polymerase activity (DNA Taq Polymerase) extends each primer, using the target nucleotide sequence as a template. The extension products are then converted into target sequences,

after dissociation of the original target strand. New primer molecules hybridise and are extended by the polymerase; the cycle is repeated to exponentially increase the number of target sequences. This technique is described in patents US 4683195 and US 4683202. Many methods for detecting and quantifying PCR amplification products have been described previously, any of which can be used in this invention. In a preferred method of the invention, the amplified product is detected by agarose gel electrophoresis as follows: five microlitres of the amplification product are separated by electrophoresis in an agarose gel at a concentration of 2%, in a TBE 0.5x buffer at 100 vdc for one hour. After electrophoresis, the gel is stained with ethidium bromide and the amplification product is observed when the gel is illuminated with ultraviolet (uv) light; as an alternative to staining, and the preferred embodiment, the amplified product can be transferred to a nylon membrane by Southern blotting techniques to be detected with a suitably labelled probe specific for the cDNA of the *FGFR3* gene.

In another example, the detection of mRNA is performed mRNA by transferring the mRNA to a nylon membrane using transfer techniques such as, for example, northern-blot, and detecting it with probes specific for the RNA or the corresponding cDNA of the *FGFR3* gene.

In a particular embodiment the amplification and quantification of the mRNA corresponding to the *FGFR3* gene are carried out by quantitative real-time RT-PCR (Q-PCR).

The final step of the method for evaluating the susceptibility of an individual of the invention comprises comparing the amount of FGFR3 protein, that of the mRNA of the *FGFR3* gene or that of the corresponding cDNA detected in a sample of an individual, with the amount of FGFR3 protein, that of the mRNA of the *FGFR3* gene or that of the corresponding cDNA detected in the samples of control subjects or in previous samples of the same individual, or with the

normal reference values.

The invention also provides an *in vitro* method for identifying and evaluating the efficacy of agents for the therapy of bladder transitional cell carcinoma, which comprises:

a) placing a culture of bladder tumour cells (with uncontrolled proliferation) in contact with the candidate compound, in the suitable conditions and for the suitable time to allow them to interact,

b) detecting and quantifying the expression levels of the *FGFR3* gene or the FGFR3 protein, and

c) comparing said expression levels with those control cultures of tumour cells.

The quantification of the expression levels of the *FGFR3* gene or the FGFR3 protein is performed in a similar manner to that described in the method of the invention for detecting *in vitro* the presence of a bladder transitional cell carcinoma in an individual.

When an agent reduces the expression levels of the *FGFR3* gene or reverses the effects of high expression of said gene, preferably reducing the levels of cell proliferation, this agent becomes a candidate for the therapy of bladder transitional call carcinoma.

Abother further aspect of this invention relates to the use of nucleotide or peptide sequences derived from the *FGFR3* gene to detect the presence of a bladder transitional cell carcinoma, to determine the stage or the severity of said carcinoma in the individual or to monitor the effect of the therapy administered to an individual with said carcinoma.

Another aspect of this invention relates to agents characterised in that they inhibit the expression and/or the activity of the FGFR3 protein. These agents, which can be identified and evaluated according to the present invention, can be selected from the group consisting of:

a) an antibody, or combination of antibodies, specific

against one or more epitopes present in the FGFR3 protein, preferably a human or humanised monoclonal antibody; also being able to be a fragment of the antibody, a single chain antibody or an anti-idiotype antibody,

b) cytotoxic agents, such as toxins, molecules with radioactive atoms or chemotherapeutic agents, including, but not limited to, small organic and inorganic molecules, peptides, phosphopeptides, antisense molecules, ribozymes, triple helix molecules etc., which inhibit the expression and/or the activity of the FGFR3 protein, and

c) antagonist compounds of the FGFR3 protein, which inhibit one or more of the functions of the FGFR3 protein

Finally, another object of the present invention is a pharmaceutical composition comprising a therapeutically effective amount of one or several of the aforementioned agents together with one or more excipients and/or carrier substances. Furthermore, said composition can contain any other active ingredient inhibiting the function of the FGFR3 protein.

The excipients, carrier substances and auxiliary substances must be pharmaceutically and pharmacologically tolerable, such that they can be combined with other components of the formulation or preparation and do not have any adverse effects on the organism treated. The pharmaceutical compositions or formulations include those that are suitable for oral or parenteral administration (including subcutaneous, intradermal, intramuscular or intravenous administration), although the best route of administration depends on the condition of the patient. Formulations can also be in the form of single doses. Formulations are prepared according to methods known in the pharmacological field. The amounts of active substances for administration can vary depending on the particularities of the therapy.

The following examples illustrate the invention.

Example 1.- Differential analysis of the expression of the

FGFR3 gene in samples of bladder tissue, using the microarrays "Human Genome U95 DNA arrays"

1.1. Materials and methods

Microarrays. GeneChip Test 3 (Affymetrix, Santa Clara) microarrays were used, which allow testing the quality of RNA before analysing expression with the GeneChip Human Genome U95A array (Affymetrix, Santa Clara), which represents 12,000 complete sequences of annotated genes; the FGFR3 gene is represented in the microarray by the set of probes 31805_at of Affymetrix, which are sense oligonucleotides with a length of 25 nucleotides, designed on the basis of the sequence Hs.1420 of Unigene, or M64347 of GeneBank (Table 1).

Table 1. Description of the probes corresponding to the set of probes 31805_at.

Consecutive order of the probes	Area of the interrogated reference sequence	Probe sequence (5'-3')	Position of the probe in the mRNA sequence of the gene
1	3511	TCCAAGCCTAAAAGGTTGTTAATAG	3227
2	3625	ATTTTTTGGACTTCAAAGCAAGCTG	3340
3	3633	GACTTCAAAGCAAGCTGGTATTTTC	3348
4	3663	AATTCTTCTAATTGCTGTGTGTCCC	3378
5	3684	TCCCAGGCAGGGAGACGGTTTCCAG	3399
6	3716	CCGGCCCTGTGTGCAGGTTCCGATG	3431
7	3722	CTGTGTGCAGGTTCCGATGTTATTA	3437
8	3821	CACTTCTTACGCAATGCTTCTAGAG	3536
9	3825	TCTTACGCAATGCTTCTAGAGTTTT	3540
10	3831	GCAATGCTTCTAGAGTTTTATAGCC	3546
11	3861	TGCTACCTTTCAAAGCTTGGAGGGA	3576
12	3873	AAGCTTGGAGGGAAGCCGTGAATTC	3588
13	3891	TGAATTCAGTTGGTTCGTTCTGTAC	3606
14	3903	GTTCGTTCTGTACTGTTACTGGGCC	3618
15	3933	TCTGGGCAGCTGTCCCTTGCTTGCC	3648
16	4005	GTGGCCAGAGGTGTCACCCAAACCG	3720

Samples. The samples studied were from biopsies, obtained by surgical transurethral resection, from control subjects without bladder transitional cell carcinoma (n=7), and from biopsies of patients that were clinically typed after resection and presented bladder transitional cell carcinoma (n=22) in one of the following stages: low-grade tumours that respect the lamina propria (Ta G1), high-grade lamina propria infiltrating tumours (T1 G3) and high-grade muscle invasive tumours (T2 G3). All the samples were histologically typed (grade and stage) in the Pathological Anatomy Department of the University Hospital Marques de Valdecilla, the same hospital where the samples has been collected, following the precepts of the Helsinki Declaration. The samples were frozen in liquid nitrogen after extraction and stored at -80°C until the time of their analysis.

Several cases were received of each type of tumour as well as of the healthy samples:

- Healthy tissue control (mucosa without muscular stratum): 5 samples
- Healthy tissue control (mucosa with muscular stratum): 2 samples
- Low-grade with lamina propria respected (TaG1): 9 samples
- High-grade lamina propria infiltrating (T1G3): 7 samples
- High-grade muscle invasive (T2G3): 6 samples

GeneChip gene expression analysis

Analysis was conducted with total RNA from individual subjects and with equimolar mixtures (pools) of total RNAs from different healthy individuals or subjects with the same stage of bladder transitional cell carcinoma. (Table 2).

Table 2. Description and number of samples analysed

	Epithelial Control	Muscular Control	Ta G1	T1 G3	T2 G3
Samples	3*(pC1)[a], 2 (pC3)	2 (pC2)	1,4(pTa.1)[b], 4 (pTa.2)	1,2(pT1.1)[c], 4 (pT1.2)	1, 2(pT2.1)[d], 3 (pT2.2)

* number of samples forming part of each pool.

[a] pC: pool of control samples. Example: 3(pC1) = pool 1 with 3 control samples.

[b] pTa: pool of samples of low-grade (G1) tumours which respect the lamina propria (Ta).
Example: 4(pTa.1) = pool 1 with 4 TaG1 samples.

[c] pT1: pool of samples of high-grade (G3) lamina propria infiltrating (T1) tumours.
Example: 2(pT1.1) = pool 1 with 2 T1G3 samples.

[d] pT2: pool of samples of high-grade (G3) muscle tissue invasive (T2) tumours.
Example: 2(pT2.1) = pool 1 with 2 T2G3 samples.

cRNA synthesis

Total RNA from each of the biopsies was obtained by homogenising the tissue in TRIzol® Reagent (Life Technologies), following the supplier's recommendations. The resulting total RNA was cleaned with the Rneasy kit (QIAGEN) (Chomczynski P. et al., Anal. Biochem., 1987, 162: 156; Chomczynski P., Biotechniques, 1993, 15: 532). Of each preparation of total RNA, 10 µg were used as starting material for synthesis of the first strand of cDNA with the reverse transcriptase enzyme SuperScript™ II RNase (Life Technologies), using as a primer an oligo-dT oligonucleotide containing the T7 phage RNA polymerase promoter sequence. The second strand of cDNA was synthesised using the enzymes DNA polymerase I of *E. coli* (Invitrogen Life Technologies), DNA ligase of *E. coli* (Invitrogen Life Technologies), RNAse H of *E. coli* (Invitrogen Life Technologies), and DNA polymerase of the T4 phage (Invitrogen Life Technologies). The biotin-

labelled cRNA was synthesised using the ENZO BioArray™ HighYield™ Transcript Labelling Kit (Enzo Diagnostics Inc). After *in vitro* transcription, the unincorporated nucleotides were eliminated using the RNeasy columns (QIAGEN).

Array Hybridization and scanning

15 µg of each biotinylated cRNA were fragmented at 94°C for 35 minutes in a buffer solution containing 40 mM Tris-Acetate (pH 8.1), 100 mM KOAc and 30 mM MgOAc. The fragmented cRNA was mixed with hybridization buffer (100 mM MES, 1M NaCl, 20 mM EDTA, 0.01% Tween 20) and heated at 99° for 5 minutes and subsequently at 45° for 5 minutes, to then be loaded in the Affymetrix array. The first array in which the hybridization was carried out was Test 3 of Affymetrix. This array allows testing the quality of RNA before analysing expression in the Affymetrix® GeneChip® Human Genome 95 A (HG-U95A).

For hybridization, arrays were incubated in a rotary oven at 45° for 16 hours with a constant rotation of 60 rpm.

Washing and staining of each array were carried out in the Affymetrix® fluid station. A washing and staining programme was used that included:

- 10 x 2 washing cycles with SSPE-T 6x (0.9 m NaCl, 60 mM NaH_2PO4, 6 mM EDTA, 0.01% Tween 20) at 25°C,
- 4x15 cycles with 0.1 mM MES, 0.1M NaCl, 0.01% Tween 20 at 50°C,
- Staining with biotinylated cRNA with a phycoerythrin streptavidin conjugate (10 µg/ml Molecular Probes)
- 10 x 4 washing cycles with SSPE-T at 25C°
- Staining an anti-streptavidin antibody for 10 minutes
- Staining a phycoerythrin-streptavidin conjugate (1 mg/ml, Molecular Probes) for 10 minutes
- 15 x 4 washing cycles with SSPE-T at 30C°

The arrays were scanned at 560 nm using a confocal microscope that uses laser emission (Agilent GeneArray

Scanner). The analysis of the intensity readings was conducted with the Microarray Suite 5.0 software. For comparison of arrays, they were scaled to a total intensity of 100.

1.2. Results

The differential analysis of the expression of the *FGFR*3 in the tumour stages with respect to the control was conducted array comparison data obtained using the Affymetrix software.. The following parameters were taken into account (in the order in which they appear in the list: i) Detection. It indicates that the transcript is Present (P), Absent (A) or Marginal (M), ii) Change: It indicates if the expression of a certain transcript Increases (I), Decreases (D), Does Not Change (NC), Marginally Increases (MI), or Marginally Decreases (MD), iii) Signal Log Ratio (SLR): It indicates the level of expression change between the base line (control) and a test sample). This change is expressed as the $\log_2$ of the ratio (*fold change* or number of times that the expression is increased or repressed in the tumour-test sample compared to the healthy-control sample). A value of SLR of 1 (equivalent to a fold change of 2), for transcripts the expression of which increases compared to the control, and of -1, for transcripts the expression of which decreases compared to the control, is considered significant.

Table 3. Results obtained for Fibroblast growth factor receptor 3 (FGFR3). Acc. N. M64347)

Control sample signal	Detection in control sample	Detection in Ta G1 stage	SLR TaG1 vs. Control	Change in TaG1 stage	Comparison
132.7	P	P	2.5	I	pTa.1 vs pC1
67.7	A	P	4.2	I	pTa.1 vs pC2
28.1	A	P	4.4	I	pTa.1 vs pC3
132.7	P	P	1	I	pTa.2 vs pC1
67.7	A	P	3	I	pTa.2 vs pC2

28.1	A	P	3.5	I	pTa.2 vs pC3
SLR Average			3.1		
Control sample signal	Detection in control sample	Detection in T1 G3 stage	SLR T1G3 vs. Control	Change in T1G3 stage	Comparison
132.7	P	P	1.7	I	pT1.1 vs. pC1
67.7	A	P	3.9	I	pT1.1 vs. pC2
28.1	A	P	3.7	I	pT1.1 vs. pC3
132.7	P	P	2	I	pT1.2 vs. pC1
67.7	A	P	4.1	I	pT1.2 vs. pC2
28.1	A	P	4.4	I	pT1.2 vs. pC3
SLR Average			3.3		
Control sample signal	Detection in control sample	Detection In T2 G3 stage	SLR T2G3 vs. Control	Change in T2G3 stage	Comparison
132.7	P	P	1.4	I	pT2.1vspC1
67.7	A	P	3.3	I	pT2.1vspC2
28.1	A	P	3.2	I	pT2.1vspC3
132.7	P	P	0.6	I	pT2.2vspC1
67.7	A	P	2.4	I	pT2.2vspC2
28.1	A	P	2.7	I	pT2.2vspC3
SLR Average			2.26		

1.3. Discussion

The differential analysis of the expression of FGFR3 gene in the tumour stages with respect to the control demonstrates that the expression levels of the FGFR3 gene were increased more than 8-fold (SLR>3) biopsies of Ta G1 low-grade or T1 G3 high-grade lamina propria invasive bladder tumours, compared to the control, whereas the increases of expression

was more than 4-fold (SLR>2) in high-grade muscle invasive tumours (T2G3) .

Example 2.- Differential analysis of expression of the FGFR3 protein in samples of bladder tissue, using the western blot technique with specific antibodies.

2.1. Materials and Methods

Samples: Samples of bladder biopsy from 3 healthy individuals (sample numbers 46, 55 and 63), six samples of low-grade superficial (Ta G1) bladder transitional cell carcinoma (sample numbers 48, 49, 50, 53, 56 and 59), three samples of high-grade lamina propria invasive (T1 G3) bladder transitional cell carcinoma (sample numbers 57, 61 and 67) four samples of high-grade muscle invasive carcinomas (T2 G3) (sample numbers 47, 51, 58 and 60) and two samples of unknown grade (sample numbers 54 and 62) were analyzed. The samples were from subjects different from those analysed with DNA microarrays. The tissues were stored at -80°C from the time they were obtained until used for extraction of proteins. All the tissues used in this study were obtained by surgical transurethral resection performed in the Urology Service of the University Hospital Marques de Valdecilla (Santander, Spain); the samples were histologically typed in the Anatomical Pathology department of the same hospital. The precepts provided in the Helsinki Declaration were followed throughout the process.

Obtaining protein extracts: The frozen samples of tissues were homogenised in mortars with liquid N_2 and RIPA B buffer (sodium phosphate 20 mM [pH 7,4], NaCl 150 mM, Triton X-100 1%, EDTA 5 mM) as well as a proteases inhibitor cocktail (Roche Diagnostics Inc., Mannheim, Germany) were added to the pulverized product.

Western blot assays: Samples were taken from the protein extracts with 20 µg of total protein, SDS-PAGE gel loading buffer with 5% β-mercaptoethanol was added to them and they were incubated at 100°C for 5 min to then load them in a 6%

polyacrylamide gel. The proteins were then transferred to nitrocellulose membranes to develop them with monoclonal antibodies directed against the extracellular domain of the FGFR3 protein (Santa Cruz Biotech. Inc., Santa Cruz, CA, USA). Finally, the membranes were hybridised with a peroxidase-conjugated secondary antibody (Amersham, Little Chalfont, UK) and the chemoluminescent signal was detected with the ECL system (Amersham). The developing films were also from Amersham. As loading control, membranes to which the proteins of gels replicated with an anti-target antibody (Amersham) were transferred.

2.2. Results.

Expression of the FGFR3 protein in bladder transitional cell carcinomas: The expression of FGFR3 in healthy samples (n=3) and tumours (n=15) was examined by western blotting. The results obtained are shown in Figure 1 and Table 4. As can be observed, the FGFR3 protein was not detectable any of the samples of healthy tissue. With regard to the tumour samples, it was present in 11 of the 15 samples analysed (73%), this percentage being higher in the low-grade tumours (83%) and the high-grade tumours that infiltrate the lamina propria (100%). The receptor appeared in the form of several immunoreactive bands of different molecular weight: 135 kDa, corresponding to the fully glycosylated form; 85 kDa, corresponding to the intracellular non-glycosylated form, and bands of intermediate molecular mass (110-110 kDa), which correspond to different degrees of glycosylation of FGFR3. Immunoreactive bands of lower molecular weight (50 kDa) were also detected that may have resulted from the proteolytic degradation thereof (Figure 1).

Table 4: Expression of FGFR-3 in bladder transitional cell carcinoma.

Sample	n	Samples positive for FGFR3	% of positive samples
Normal bladder	3	0	0
Ta G1	6	5	83
T1 G3 Carcinoma	3	3	100
T2 G3 Carcinoma	4	2	50
Unknown grade carcinoma	2	1	100

2.3. Discussion

The results set forth herein show that the FGFR3 protein, absent in normal bladder, is expressed in most bladder transitional cell carcinomas. In some said tumours the levels of FGFR3 protein are singularly high. The sensitivity of the detection system is 73% and the specificity is 100%.

Example 3. Inhibition of cell proliferation in bladder tumour cell lines treated with antibodies specific against the FGFR3 protein.

3.1. Materials and Methods

Culture cell lines: The bladder transitional cell carcinoma line used in this study was RT112 (Marshall, C.J., et al., J. Natl. Cancer Inst., 1977, 58:1743), derived from a primary tumour; which was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, RFA). The RT-112 line was grown in RPMI medium, supplemented with 10% FBS and 2 mM glutamine, except where otherwise indicated. All the tissue culture reagents (media, serum, etc.) were obtained from Invitrogen (Paisley, UK).

Preparation of Protein Lysates: Cells from a 10 cm plate were washed twice with cold PBS and collected in 0.5 ml of RIPA B. Samples were then centrifuged at 15000 x g for 10 min at 4°C to remove cell remains, the supernatant was recovered and the protein concentration was estimated by means of the Bradford assay (BioRad, Hercules, CA, USA) (Molina, M. A. et al., Cancer Res., 1999, 59: 4356-4362).

Western blot assays: Samples were taken from the protein extracts with 20 µg of total protein, SDS-PAGE gel loading buffer with 5% β-mercaptoethanol was added to them and they were incubated at 100°C for 5 min to then load them in a 6% polyacrylamide gel. The proteins were then transferred to nitrocellulose membranes to develop them with monoclonal antibodies directed against the extracellular domain of FGFR3 (Santa Cruz Biotech. Inc., Santa Cruz, CA, USA). Finally, the membranes were hybridised with a peroxidase-conjugated secondary antibody (Amersham, Little Chalfont, UK) and the chemoluminescent signal was detected with the ECL system (Amersham). The developing films were also from Amersham. As a control of the amount of loaded protein, membranes, to which the proteins of gels replicated with an anti-actin antibody (Amersham) were transferred, were hybridised.

Cell Proliferation Assays: The effects of mouse monoclonal antibodies against FGFR3 and β2-microglobulin on cell growth were determined by means of proliferation assays. The antibodies were concentrated and washed three times with PBS using a 10 kDa Amicon filter (Millipore CO., Bedford, MA), to thus remove the sodium azide with which they are supplied. They were then sterilized by passing them through a 0.2 µm filter previously saturated with DMEM+10% FBS and they were diluted in culture medium. The RT-112 cells were seeded at a density of 2000 cells per well in 96-well plates in RPMI+10% FBS medium. The cells were allowed to adhere for 24 hours and the medium was then removed to replace it with fresh RPMI with antibody concentrations between 0 and 20 mg/ml. After an incubation of 1 or 2 days, the number of cells was estimated by means of the MTT (methylthiazoltetrazolium) assay (Sigma Chemical Co., St Louis, USA) as described below. A 1 mg/ml solution of MTT (methylthiazoltetrazolium) min culture medium with serum was prepared. After the incubation of 1 or 2 days, the medium was removed from the wells and 100 ml of the medium

with MTT were added. Some wells without cells were used as blanks. The plate was incubated at 37°C between 30 minutes and one hour. After this time, a purplish precipitate is formed inside the cells, which is often visible. The medium was then removed and 100 ml of non-sterile DMSO per well were added. The absorbance of the well was determined at 550 nm and the amount of cells of each well was extrapolated using a standard curve.

3.2. Results.

Expression of the FGFR3 protein in the bladder transitional carcinoma cell line RT-112: The expression of FGFR3 was studied by means of western blot analysis, high levels of the receptor being detected (Figure 2). This is observed in the form of various immunoreactive bands of different molecular weight: 135 kDa, corresponding to the fully glycosylated form; 85 kDa, corresponding to the intracellular non-glycosylated form, and bands of intermediate molecular mass (100-110 kDa), which correspond to different degrees of glycosylation of FGFR3. Immunoreactive bands of lower molecular weight (50 kDa) were also detected that may have resulted from the proteolytic degradation thereof.

Inhibition of cell growth by antibodies against FGFR3: During recent years, many antibodies have been described that are directed against extracellular domains of membrane receptors with antiproliferative properties. For this reason, it was decided to test whether anti-FGRF3 monoclonal antibodies were capable of inhibiting the growth of bladder transitional carcinoma cells in culture. For the assays, the line RT-112, the only one expressing detectable levels of the receptor, was chosen. The assays were performed in serum-free medium or in medium supplemented with 10% FBS and the cells were incubated in the presence of antibody for 24 and 48 hours. As a control, also monoclonal mouse anti-β2 microglobulin antibodies, supplied by the same company (Santa Cruz) were used at the same concentration as the anti-FGFR3

antibodies. It was seen that the azide with which said antibodies are supplied was toxic for the RT-112 cells, therefore it was removed by means of successive concentrations with 10 kDa Amicon type filters. Thus, only antibodies from which the azide had been removed were used for the proliferation assays. Furthemore, it was previously assayed by means of Western blot that they had not lost activity during the concentration and filtration process. As can be observed in Figure 3, the anti-FGFR3 antibodies inhibited the proliferation of RT-112 cells in serum free-medium after 2 days of treatment, while the anti-β2 microglobulin antibodies had no effect. On the other hand, if the assays were carried out in medium with 10% FBS, none of the antibodies had significant effects on the proliferation of the RT-112 cells.

3.3. Discussion

The results set forth herein show that the expression levels of the FGFR3 protein, absent in normal bladder, are high in the human bladder transitional cell carcinoma line RT-112. FGFR3 is a membrane glycoprotein that binds to growth factors of the FGF family, triggering an intracellular signal cascade stimulating cell proliferation (Keegan et al., Oncogene, 1991, 6:2229-2236). Said receptor could thus have a role in the origin and progression of bladder transitional cell carcinoma.

The treatment of RT-112 cells with a monoclonal antibodies directed against the extracellular domain of FGFR3 inhibits the growth thereof in the absence of serum. Several non-mutually exclusive mechanisms could explain this effect: the antibody could block the binding site for the growth factor or prevent the dimerisation of the receptor (before its activation), or cause the depletion thereof from the plasma membrane.

In short, the over-expression of FGFR3 in bladder transitional cell carcinoma and the fact that antibodies against it inhibit the proliferation of transitional carcinoma

cells in culture makes it a good candidate for a therapeutic target for the development of drugs against bladder transitional cell carcinoma; these same results show that the active ingredient of one of those drugs could be an antibody specific against FGFR3 .

CLAIMS

1. An *in vitro* method for detecting the presence of a bladder transitional cell carcinoma in an individual, for determining the stage or the severity of said carcinoma in the individual or for monitoring the effect of the therapy administered to an individual with said carcinoma, which comprises:

a) the detection and/or quantification of the FGFR3 protein, of the mRNA of the FGFR3 gene or the corresponding cDNA in a sample of an individual, and

b) the comparison of the amount of FGFR3 protein, of the amount of mRNA of the *FGFR3* gene or of the amount of the corresponding cDNA detected in a sample of an individual, with the amount of FGFR3 protein, with the amount of the mRNA of the *FGFR3* gene or with the amount of the corresponding cDNA detected in the samples of control individuals or in previous samples of the same individual or with the normal reference values.

2. The method according to claim 1, wherein said sample is a sample of bladder tissue.

3. The method according to claim 3, wherein said sample of bladder tissue to be analysed is obtained by any conventional method, preferably cystoscopy.

4. The method according to claim 1 and 2, wherein said sample is a sample of urine, blood, plasma, serum, pleural fluid, ascitic fluid, synovial fluid, bile, semen or cerebrospinal fluid.

5. The method according to claim 1, wherein said sample to be analysed is obtained from an individual not previously diagnosed with bladder transitional cell carcinoma.

6. The method according to claim 1, wherein said sample to be analysed is obtained from an individual who has been previously diagnosed with bladder transitional cell carcinoma.

7. The method according to claim 1, wherein said sample to be analysed is obtained from an individual receiving

treatment, or who has been previously treated for bladder transitional cell carcinoma.

8. The method according to claim 1, characterised in that it comprises an extraction of the sample, either to obtain an extract of proteins or to obtain an extract consisting of total RNA.

9. The method according to claim 8, characterised in that the detection of the FGFR3 protein comprises a first step for placing the extract of proteins of the sample in contact with a composition of one or more antibodies specific against one or more epitopes of the FGFR3 protein, and a second step for quantifying the complexes formed by the antibodies and the FGFR3 protein.

10. The method according to claim 9, characterised in that said antibodies comprise monoclonal antibodies, polyclonal antibodies, intact or recombinant fragments thereof, combibodies and Fab or scFv fragments of antibodies, specific against the FGFR3 protein; these antibodies being human, humanised or of non-human origin.

11. The method according to claims 9 or 10, characterised in that for the quantification of the complexes formed by antibodies and the FGFR3 protein, techniques are used which are selected from the group consisting of: western-blot, ELISA (Enzyme-Linked Immunosorbent assay), RIA (Radioimmunoassay), Competitive EIA (Competitive Enzyme Immunoassay), DAS-ELISA (Double Antibody Sandwich-ELISA), immunocytochemical or immunohistochemical techniques, techniques based on the use of biochips or protein microarrays that include specific antibodies, assays based on the precipitation of colloidal gold in formats such as dipsticks; or by means of affinity chromatography techniques, ligand binding assays or lectin binding assays.

12. The method according to claim 8, characterised in that the detection of the mRNA or of the corresponding cDNA of the *FGFR3* gene comprises a first step for amplifying the mRNA,

included in the extract of total RNA, or the corresponding cDNA synthesised by reverse transcription of the mRNA, included in the extract of total RNA; and a second step for quantifying the amplification product of the mRNA or of the cDNA of the *FGFR3* gene.

13. Method according to claim 12, characterised in that the amplification is performed qualitatively or quantitatively by means of RT-PCR using oligonucleotide primers, the sequences of the primers used being 5'-GACGGTTTCC AGGGAGGGGC-3' and 5'-GTAACAGTACAGAA CGAACCAACTG-3'.

14. The method according to claim 8, characterised in that the detection is carried out with probes specific for the mRNA or the corresponding cDNA of the *FGFR3* gene, by means of techniques such as northern-blot.

15. The method according to claim 8, characterised in that the detection of the mRNA is carried out by means of quantitative real-time RT-PCR (Q-PCR).

16. Use of nucleotide or peptide sequences derived from the *FGFR3* gene to detect *in vitro* the presence of a bladder transitional cell carcinoma in an individual, to determine *in vitro* the stage or the severity of said carcinoma in the individual, or to monitor *in vitro* the effect of the therapy administered to an individual with said carcinoma.

17. An *in vitro* method for identifying and evaluating the efficacy of compounds for the therapy of bladder transitional cell carcinoma, which comprises:

a) placing a culture of bladder tumour cells (with uncontrolled proliferation) in contact with the candidate compound, in the suitable conditions and for the suitable time to allow them to interact,

b) detecting and/or quantifying the expression levels of the *FGFR3* gene or the FGFR3 protein, and

c) comparing said expression levels with those of the control cultures of tumour cells not treated with the candidate compound.

18. Use of nucleotide or peptide sequences derived from the *FGFR3* gene, in methods for screening for, identifying, developing and evaluating the efficacy of compounds for the therapy of bladder transitional cell carcinoma.

19. Agents characterised in that they inhibit the expression and/or the activity of the FGFR3 protein.

20. The agents according to claim 19, selected from the group consisting of:

a) an antibody, or combination of antibodies, specific against one or more epitopes present in the FGFR3 protein, preferably a human or humanised monoclonal antibody; also being able to be a fragment of the antibody, a single chain antibody or an anti-idiotype antibody,

b) cytotoxic agents, such as toxins, molecules with radioactive atoms or chemotherapeutic agents, including, but not limited to, small organic and inorganic molecules, peptides, phosphopeptides, antisense molecules, ribozymes, triple helix molecules, double stranded RNA etc., which inhibit the expression and/or the activity of the FGFR3 protein and

c) antagonist compounds of the FGFR3 protein, which inhibit one or more of the functions of the FGFR3 protein.

21. The agents according to claims 19 or 20 for the treatment of bladder transitional cell carcinoma.

22. Use of the agents according to claims 19 or 20 in the manufacture of a medicinal product for the treatment of bladder transitional cell carcinoma.

23. A pharmaceutical composition comprising a therapeutically effective amount of one or several agents according to claims 19 or 20 together with at least one pharmaceutically acceptable excipient.

24. The pharmaceutical composition according to claim 23, characterised in that it contains another active ingredient, preferably one that inhibits the function of the FGFR3 protein.

Figure 1

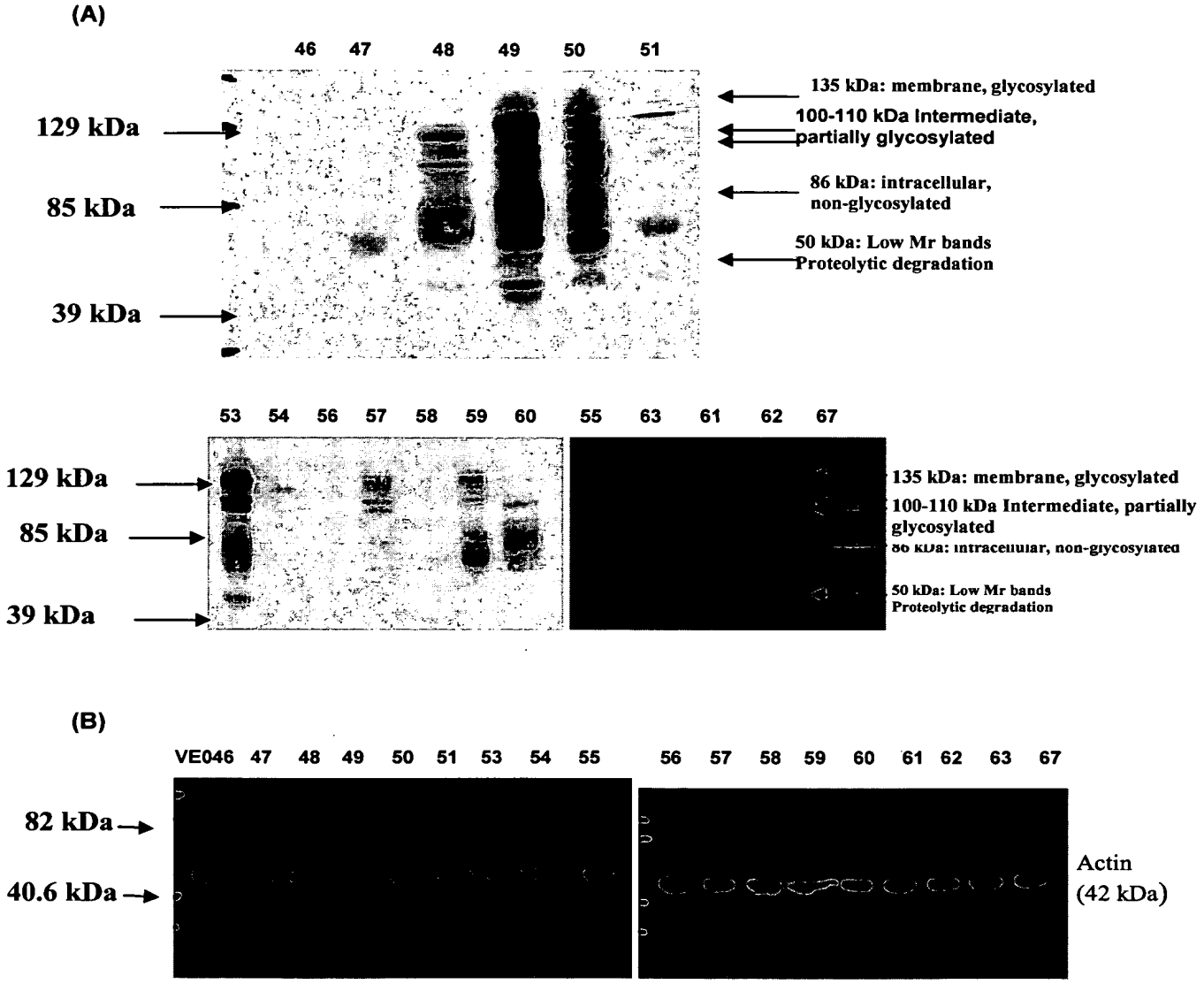


Figure 2

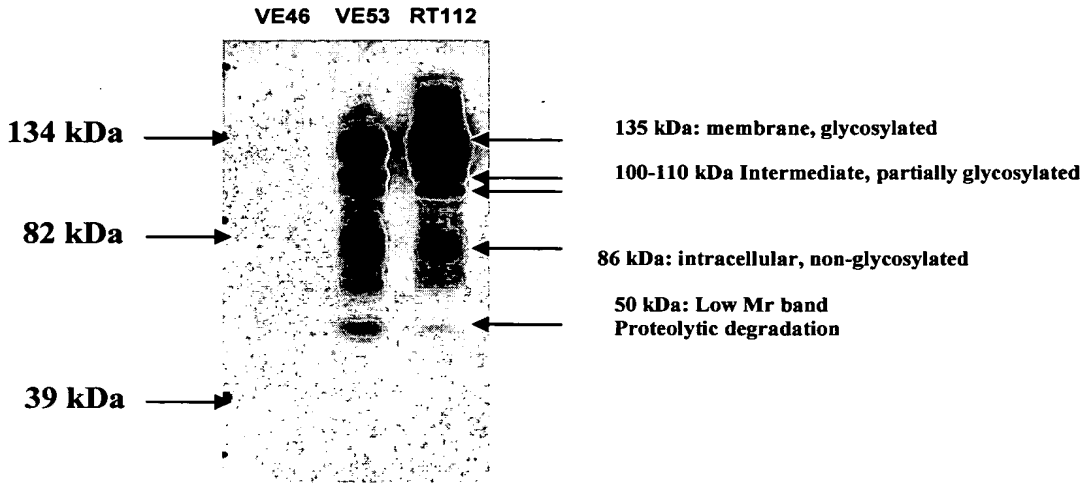
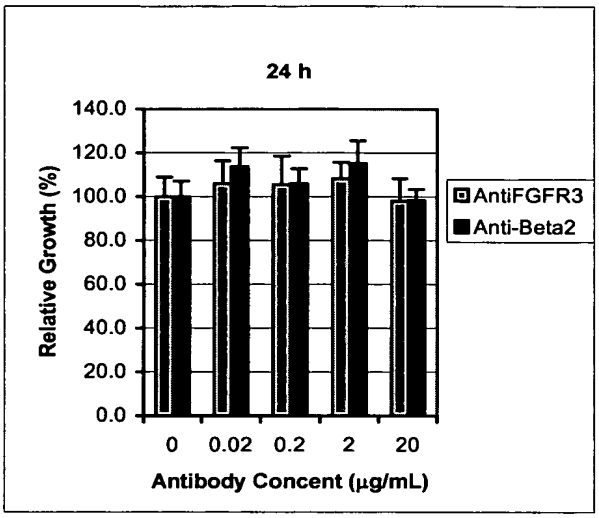
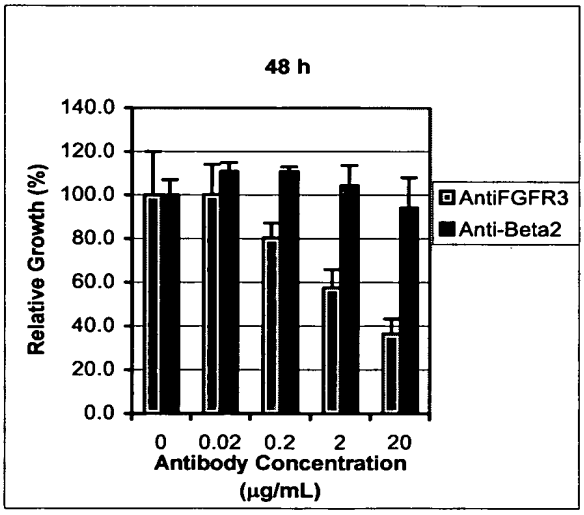


Figure 3





I, Catalina Mora Estevan, Sworn Translator duly appointed by the Ministry of Foreign Affairs of Spain, do hereby certify that the foregoing is a true and complete translation into English of the original Spanish document.

Madrid, 12th July 2010

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